MORPHOGENESIS OF EPITOKOUS SETAE DURING NORMAL AND INDUCED METAMORPHOSIS IN THE POLYCHAETE ANNELID NEREIS GRUBEI (KINBERG)[1]

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Most species of nereid polychaetes end their lives by undergoing a complex metamorphosis in preparation for a single brief period of reproductive activity. The metamorphosis is completed at the time of sexual maturity. At this time, having acquired modifications which adapt them to a pelagic existence, these formerly benthic animals leave their mucus tubes and swarm in midwater, where mass spawning takes place.

Although the production of paddle-shaped "pelagic" setae is one of the most characteristic features of nereid metamorphosis, the origin of the chaetogenic tissue has not yet been completely described. The characteristic setae of the benthic atoke are replaced throughout the posterior region with an orderly array of heteronereid setae. These greatly increase the effective surface area of the parapodium and probably serve as oars during the brief pelagic existence of the mature worm. Heteronereid chaetogenesis has been described in the European *Perinereis cultrifera* by Bauchot-Boutin and Bobin (1954). The author has observed a similar series of events in *Nereis grubei*, but the observation of earlier stages now requires a new interpretation of the origin and nature of the chaetogenic tissue.

Nereid metamorphosis is known to be regulated by an inhibitory hormone produced by cells in the supra-esophageal ganglion (Durchon, 1952), but no attempt to define the response of individual somatic tissues to hormone deprivation has yet been reported. The information reported here on the earliest stages in the process of chaetogenesis permits an accurate interpretation of the response of parapodial epidermis to the removal of the inhibitory hormone at different stages of normal development.

Materials and Methods

Specimens of *Nereis grubei* were collected from burrows in the holdfasts of the brown kelp *Egregia menziesii* and from the sandy substrate beneath the abundant red alga *Gasteroclonium coulteri* at Pescadero Point, Monterey County, California.

Control animals were sacrificed immediately after oöcyte measurements had been taken. Experimental animals were maintained in deep Petri dishes in filtered, antibiotic-containing sea water (terramycin, 25 mg./l. or a combination of penicillin, 10^6 units/l. and streptomycin, 100 mg./l.). The temperature was maintained at 15–16° C. by flow of cool sea water around the bases of the Petri dishes.

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Oöcyte diameters were determined by removing a sample of coelomic contents from about segment 30 of worms anesthetized in 7.3% $MgCl_2$. Each sample was removed in a disposable capillary micropipette, deposited in a drop of mineral oil on a circular coverslip, and examined as a hanging drop preparation. Diameters of 10–15 oöcytes, including all sizes present, were recorded for each animal, and in those cases where the oöcytes present appeared to include two distinct size classes, the average diameter of the largest size class was used to characterize the animal (Schroeder, 1966). In instances where two size classes were not apparent, all the oöcytes from a given animal were averaged. The diameters were all measured from living cells with the same binocular microscope.

Rates were determined by sequential measurements from animals maintained in the laboratory without feeding.

Decapitation involved the removal of the prostomium and the peristomial tentacles from anesthetized animals with fine Wecker scissors. In most cases the proboscis of the experimental animals was not injured and the wound healed well. Previously anesthetized animals were fixed in sea water Bouin's fluid. After fixation, segments 44–46 were cut from each specimen, imbedded in 61° paraffin and sectioned at 5–8 μ. Although no autoradiographic observations are reported here, most of the slides upon which this study is based were autoradiograms of serial cross sections. The slides were dipped in Ilford K5 Liquid Nuclear Emulsion, exposed for 2–4 weeks in a dark, dry atmosphere, developed in Kodak D-19 and stained with Mayer's hemalum and celestine blue (Doniach and Pelc, 1950). Some slides were stained with Ehrlich's hematoxylin and triosin, Mallory's triple stain, and Heidenhain's Azan procedure.

The terms "chaeta" and "seta" have been considered synonymous, and are used interchangeably herein. "Seta," the Latin form, has been preferred as a noun; "chaeta," the Greek form, has been preferred in combinations.

Morphogenesis of Heteronereid Setae

The appearance of the tissue which will ultimately produce the heteronereid setae is among the earliest detectable events of metamorphosis. Its initial formation occurs when the major oöcyte size class reaches a diameter of approximately 95 μ. The subsequent development of the chaetogenic tissue may be divided into four stages, which are summarized in Table I. Separation of the first three stages

Table I

Stages in the development of the heteronereid setal sacs

Stage	Characteristics	Oöcyte diameter	Duration*
I	Movement of proliferated epidermal cells along atokous setal sac; differentiation of first chaetoblast cells	95–130 μ	*ca.* 22 days
II	Appearance of paddles within the chaetogenic tissue	130–165 μ	*ca.* 22 days
III	Appearance of setal shafts within the chaetogenic tissue	165–180 μ	*ca.* 9 days
IV	Eruption of fully formed setae to the exterior	over 180 μ	*ca.* 13 days

* Durations are calculated from an oöcyte growth rate of 1.6 μ/day and are approximate. The duration of stage IV is especially likely to be variable. (See text.)

depends upon observation of the characteristic sculpturing of different portions of the heteronereid setae with phase contrast optics (Fig. 1). The first stage includes the period of proliferation and is terminated at about the time that the oöcytes reach a diameter of approximately 130 μ. At this point, about 22 days after the initiation of stage I, the first signs of the paddles (the terminal portions of the compound setae) make their appearance within the chaetogenic tissue. Stage II comprises the period during which only the paddles may be detected and also lasts about 22 days. When the oöcytes are 165–170 μ in diameter, the shafts appear within the chaetogenic tissue. The presence of these shafts defines stage III, which lasts until the paddle-ends erupt through the epidermis to the exterior, a period of about 9 days. After eruption, which occurs when the oöcytes are approximately 180 μ in diameter, the setal sacs are in the final stage IV, where they remain for approximately two weeks while metamorphosis is completed. Since stage definition rests upon unambiguous morphological criteria, the stage of any metamorphosing specimen may be accurately determined, even from cleared and stained whole mounts of parapodia.

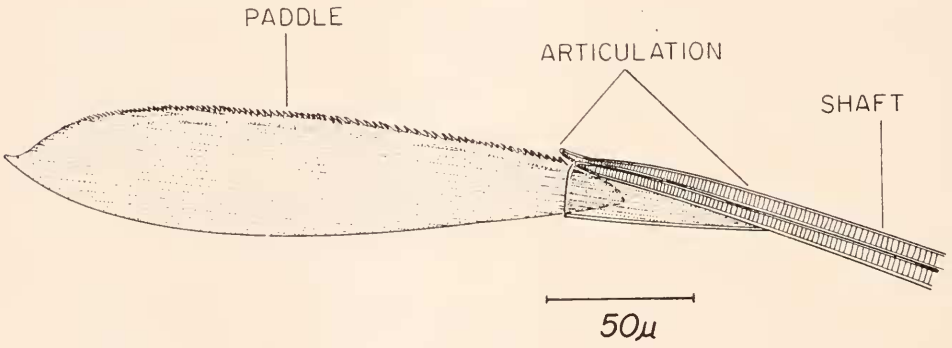


FIGURE 1. The structure of a heteronereid seta, illustrating the characteristic sculpturing of the components.

Setal sac development was found to progress somewhat independently in the dorsal and ventral portions of the parapodium (the notopodium and the neuropodium, respectively). The notopodium is occasionally found to be in a stage slightly more advanced than the neuropodium; and the slightly more rapid development of the heteronereid setae in the notopodium is probably a regular feature of setal sac development.

Stages in setal sac morphogenesis

Stage I

New chaetogenic tissue appears to arise by proliferation from the epidermis at two sites on the parapodium of the atoke. These areas are indicated in Figure 2 at the bottom of deep folds just dorsal to the notopodial setal bundle and ventral to the neuropodial setal bundle. The tissue is especially prominent in autoradiograms stained with Mayer's hemalum and celestine blue. Proliferating cells spread

over the surface of the atokous setal bundles, which are separated from the coelom by an aniline blue-positive peritoneum. Peritoneal nuclei may also be seen lining the coelomic surface of the new chaetogenic tissue, which inserts itself between the atokous setal sac and its peritoneal covering. The most characteristic cells of the new tissue, thought to represent chaetoblasts, have large nuclei with one to three prominent nucleoli. With the stain used, the boundaries of the cells could not be distinguished, and Figure 2 shows the tissue as it appears in both the notopodium and the neuropodium.

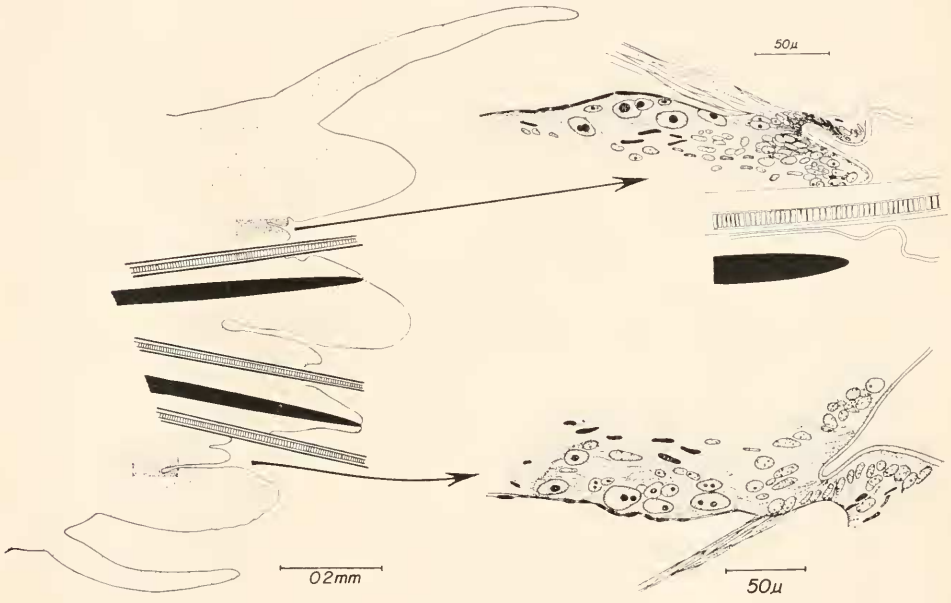
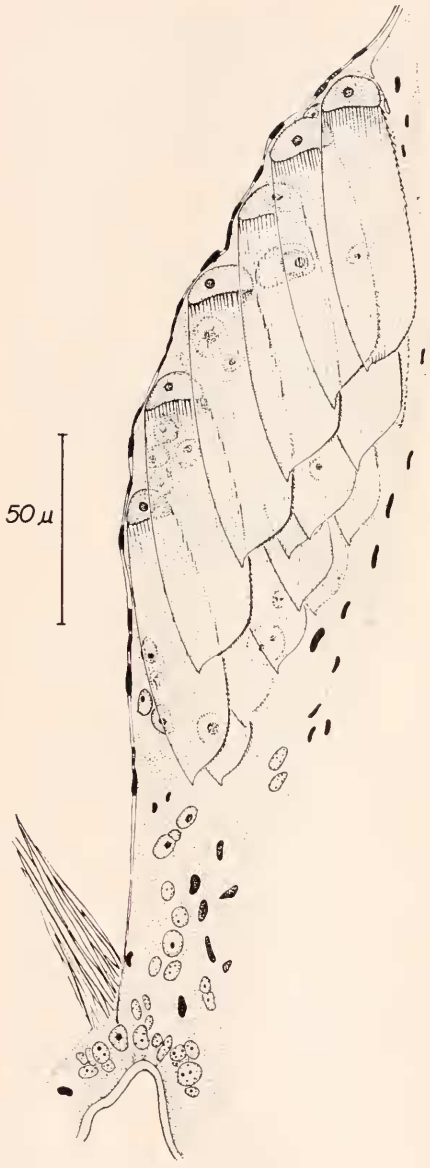


FIGURE 2. An outline of an atokous parapodium. The chaetogenic areas for the notopodial and neuropodial heteronereid setae are stippled. Insets: the appearance of the earliest stage of development of the heteronereid setae. Upper: stage I, notopodium. Lower: stage I, neuropodium.

Setal sacs in stage I can be detected in parapodial whole mounts. After staining with an alum hematoxylin and clearing in toluene, the new tissue appears as a pair of basophilic mounds on the dorsal surface of the notopodial setal sac and the ventral surface of the notopodial setal sac.

Stage II

Stage II is defined by the presence of paddles, which are the blade-like terminal portions of the heteronereid setae. Figures 1 and 3 show the characteristic fine striations which parallel the longitudinal axis of the blade. These are best seen



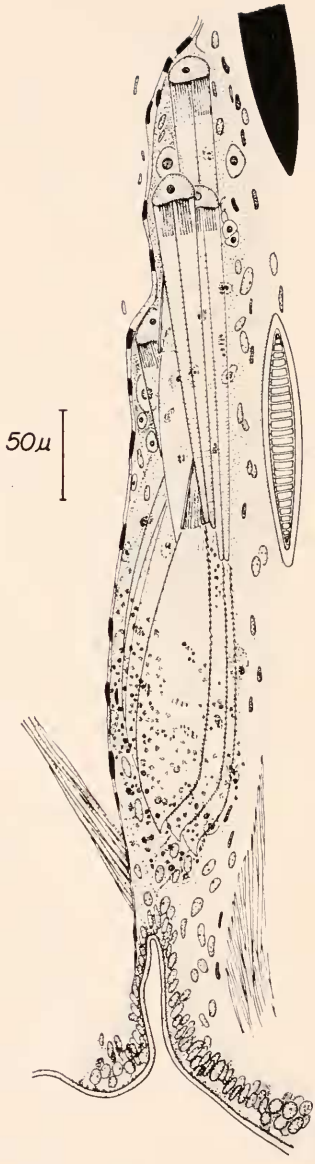


FIGURE 3. Diagrammatic longitudinal section of a neuropodial setal sac in stage II.
FIGURE 4. Diagrammatic longitudinal section of a neuropodial setal sac in stage III.
A cross section of an entire worm yields longitudinal sections of the parapodia, from which these diagrams were prepared.

with phase contrast optics, which permit the detection of even small portions of paddles in unfavorable sections. The paddles are secreted by prominent chaetoblast cells at the base of the developing setal sac. The sculpturing appears to be molded by a well-developed "ciliary" border (*appareil ciliaire*) along the secretory edge of the chaetoblast (Bauchot-Boutin and Bobin, 1954), which is indicated in Figure 3 by thickened lines of sculpturing at the base of the paddle. The paddles are stacked closely together, but stainable material and occasional prominent nuclei are present between them. A tenuous connection with the epidermis is retained. Although no further cells are contributed to the setal sac once paddle production is underway, the connection may be identified by its greater basophilia.

Stage III

The appearance of the shafts, with their characteristic double column sculpturing at right angles to the setal axis (Figs. 1 and 4), provides a convenient and unambiguous criterion for the determination of stage III. The shafts of the atokous setae are sculptured with but a single column of short horizontal bands (Figs. 2 and 4). The presence of two such columns in the shaft of the heteronereid seta provides a ready distinction between the two types in sections, even when the terminus of the setae is lost. Near the articulation of the atokous setae the columns are occasionally multiplied, so that comparison must be made between portions of the shaft removed from the articulation.

The paddles reflect light brilliantly, and, when well-developed, may be seen within the parapodia of living specimens. Stages II and III may not be distinguished, however, without examination of an isolated parapodium under higher magnification.

Stage IV

Stage IV is also defined by an unambiguous event, the eruption of the paddle ends through the parapodial epidermis. The point of eruption is readily found in a series of sections. It usually occurs when the oöcytes are about 180 μ in diameter, and swarming is estimated to follow in about 13 days.

It seems probable that the initiation of stage I is coincident with the increase in the growth rate of the oöcytes. The average rate of growth of oöcytes over 100 μ in diameter is about 1.6 μ/day, and is distinct from the much slower growth rate of smaller oöcytes (Clark and Ruston, 1963; Schroeder, 1966). Since the range of oöcyte diameters is known for each stage, the duration of each stage has been calculated and included both in above description and in Table I. These values add to a total of 67 days for metamorphosis of the setal sacs and thus perhaps for the interval between the first waning of the level of the inhibitory hormone and actual swarming. Specimen G135, taken from the field with 111 μ oöcytes, swarmed 62 days later in the laboratory. Using the total given above, corrected for the late start, swarming would have been predicted after 58 days.

It should be recalled that the above timetable applies to segments 44–46. The timetable of setal sac morphogenesis, and especially its correlation with oöcyte growth, varies along the length of the heteronereid region (Reish, 1954).

Effects of Decapitation on Setal Sac Development

Removal of the prostomium deprives nereids of the hormone which inhibits metamorphosis, and therefore induces metamorphic changes prematurely. An analysis of the relationship between the development of the setal sacs and the presence of the hormone has been possible because of the well-defined stages into which the process can be divided, and because information on the oögenesis of the species is available (Schroeder, 1966).

Figure 5 illustrates normal setal sac development in relation to oöcyte growth, and the range of oöcyte diameters found during each normal setal sac stage is

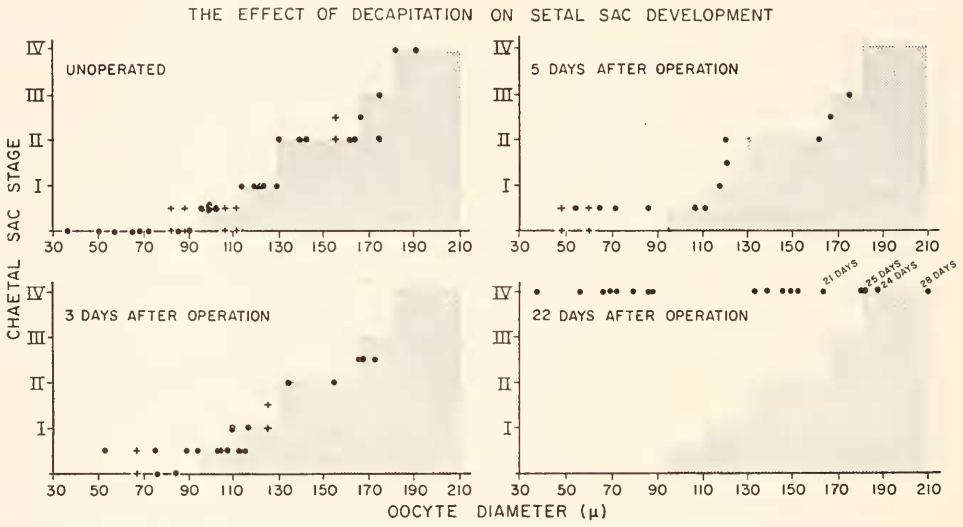


Figure 5. The development of the heteronereid setal sacs and their response to decapitation. For specimens in which the notopodium is in a slightly more advanced stage than the neuropodium, the point for the animal has been split into two crosses, the upper of which designates the neuropodium in all cases. In order to give a clearer picture of development, animals which had obviously just entered upon a given stage are reported as being midway between the two stages on the graph. The periods of normal development have been indicated on the graphs of the experimental results to facilitate their comparison with normal development. The points for experimental animals with oocytes over 95 μ original diameter have been corrected as described in the text.

indicated. The normal duration of each setal sac stage has also been indicated by the stippled areas on the graphs (Fig. 5) of the experimental results, to facilitate their comparison with normal development. Since the average oöcyte growth rate is known, this figure has been used to correct the data presented for operated animals of oöcyte diameter greater than 95 μ. Thus when an animal with 100 μ oöcytes is decapitated and maintained for 22 days, it can be predicted that with normal growth the oöcytes would have been approximately 135 μ in diameter at the end of the 22 days, and that correspondingly the setal sacs ought to be in stage II. They are in fact found to be in stage IV, and their development has

clearly been accelerated. Such corrections have not been made for animals with oöcyte diameters under 95 μ since the absolute value of the oöcyte growth rate in these animals is less well established. It should also be noted that in some young animals a few deeply basophilic cells are present which represent the earliest appearance of stage I. These animals have been represented in the graphs as a partial stage I, since they include many fewer cells than are illustrated in Figure 2. In a similar manner, the earliest appearance of the paddles has been noted as a partial stage II, and the earliest appearance of the shafts as a partial stage III, in order to provide a better picture of the progress of development.

The effect of decapitation upon the development of the heteronereid setae has been examined in a series of animals of increasing age. Decapitation of gameteless animals induced the formation of a few small, relatively unsubstantial heteronereid setae. The animals were examined 15 or more days after decapitation, and 11 of 12 animals had produced the tiny "pelagic" setae. Since the setae thus unnaturally produced are smaller and more transparent and not as readily found as normal heteronereid setae, it is probable that they had been produced but were not observed in the one exceptional individual.

Decapitation of animals with oöcytes under 95 μ in diameter, which have not yet begun to develop heteronereid setae, also induces the formation of a few tiny "pelagic" setae. These were observed 22 days after decapitation in 7 out of 8 animals, whose oöcytes ranged from 37 μ to 88 μ in average diameter. Additional animals with oöcytes under 95 μ were examined 3 and 5 days after decapitation (Fig. 5). In 4 of the 6 animals examined 3 days after decapitation recognizable chaetoblast cells were present, although not as many as in the full stage I (Fig. 2). After 5 days, such chaetoblasts were present in at least one portion of the parapodium of all 6 of the animals examined. No traces of further development were found after 5 days, although after 22 days the differentiation and production of heteronereid setae is complete. Thus, at least a few cells of the parapodial epidermis appear to be competent to differentiate in response to hormone deprivation, even in very young worms.

Results obtained by decapitation of animals with oöcytes greater than 95 μ in diameter and in which normal production of the heteronereid setal sacs has begun, are also reported in Figure 5. The oöcyte sizes indicated for the experimental animals in this oöcyte size range have been corrected to account for normal oöcyte growth. Animals sacrificed 3 and 5 days after decapitation demonstrate very little acceleration of setal sac morphogenesis. Only two of these specimens, both stage I animals sacrificed 5 days after decapitation, appeared to show a response to the operation. The 22-day experiments clearly indicate that decapitation does yield accelerated development when performed on animals in stages I and II. It seems likely that the first two stages last too long to permit the histological resolution of changes induced in them over short periods.

For various reasons, a number of animals was sacrificed at irregular intervals after decapitation, and data from 14 such cases are summarized in Table II, arranged in the order of increasing oöcyte diameter. For those with oöcyte diameters above 95 μ, estimates of normal oöcyte growth and setal sac stage for the post-operative period involved are included. Acceleration of setal sac development is clearly shown in animals with oöcytes up to 140–145 μ, even after only

TABLE II

Decreasing response of setal sac development to hormone deprivation during metamorphosis

Animal no.	No. of days	Average oöcyte diameter (μ)		Setal sac stage	
		Initial	Expected	Expected	Observed
G375	17	56			Notopodium IV Neuropodium 0
G403	13	108	129	I–II	IV
G153	18	115	138	II	IV
G188	7	130	141	II	III
G160	8	132	145	II	III
G168	7	138	149	II	III
G241	15	140	164	II–III	III
G165	4	143	149	II	II
G436	13	159	180	III–IV	early III
G437	4	161	167	II–III	III
G207	6	162	172	III	III
G205	5	167	175	III	III
G206	6	169	179	III–IV	III
G458	13	170	191	IV	III

TABLE III

A concordance of stages of post-larval development in epitokous nereid polychaetes

Proposed stage		Author's designation	Characteristic of stage
1		Durchon 0 Hauenschild 3	Atokous, without gametes
2		Charrier A Hauenschild 2	Atokous, with oöcytes growing at slower rate. No heteronereid chaetoblasts present
3	Hauenschild 1	Setal Sac I	Differentiation of first chaetoblasts; increase in oöcyte growth rate; probable decrease in hromone level
—		Durchon I	Shortening of segments; increased vascularization
4		Setal Sac II	Internal appearance of heteronereid paddles
—		Charrier B Durchon II	Enlargement of parapoidal lobes
5		Setal Sac III	Internal production of heteronereid shafts
—		Charrier C	"Heteronereis without swimming setae"
6		Durchon III Setal Sac IV	Eruption of heteronereid setae from parapodia
7		Charrier D Durchon IV Hauenschild 0	Oöcytes with mature morphology; loss of atokous setae; completion of parapodial transformation

seven days. Specimens with larger oöcytes do not show acceleration, and two do not even reach the stage expected. When comparing this table to the results reported in Figure 5 it should be recalled that the oöcyte diameters given there for animals with an initial oöcyte diameter over 95 μ have been corrected, and correspond to the expected oöcyte diameters in Table II. The hormone level is presumably waning during metamorphosis and a point is probably reached at which the hormone level is so low that it no longer influences setal sac morphogenesis.

Stages of Nereid Post-Larval Development

The stages in the development of the heteronereid setae provide a more precise and accurate basis for subdividing the period of nereid metamorphosis than has heretofore been available. The stages previously reported by Charrier (1920) and Durchon (in Bauchot-Boutin and Bobin, 1954) are based upon the appearance of external features which cannot be defined with precision. Table III is an attempt to correlate the stages described by these authors, the setal sac stages defined herein, and the broader, more physiologically defined stages of Hauenschild (1966), into a series of stages which will define the whole of the post-larval existence of an epitokous nereid. Examination of a female specimen should permit its assignment to any of the seven periods, although the sacrifice of a parapodium may be required to accomplish this most accurately if the oöcytes are sufficiently well-developed. The stages may be applied to males, but the events of spermatogenesis have not yet been correlated with setal sac development.

Discussion

The development of the heteronereid setae was studied by Bauchot-Boutin and Bobin (1954) from cross sections of the parapodia of *Perinereis cultrifera*. Although what they saw in this species does not appear to differ essentially from the observations on *N. grubei*, they did not observe the earliest stage of the process and interpreted the origin of the chaetogenic tissue to be different from what has been seen in *N. grubei*. They concluded that the heteronereid setae were produced from a germinal layer of chaetoblasts located on the external border of the setal sac, which had formerly produced replacement setae for the atoke. They were primarily interested in showing that the changeover to production of the new setal type is accomplished by a mechanism different from that involved in the regeneration of simple setae in sabellids, in which an injured setal sac degenerates and is replaced with new chaetogenic tissue before a replacement seta is produced (Bobin, 1947). The apparent unavailability of the earliest stages of heteronereid chaetogenesis forced them to commence their examination at last in setal sac stage II, since paddles are unquestionably present in their illustration. In fact, the chaetoblasts which are producing these setae do arise from a new epidermal proliferation. Although both nereid and heteronereid setal sacs are present simultaneously, there is a gradual reduction in the volume of the atokus chaetal sac. The vacated space is occupied by the developing heteronereid tissue. Since the animal retains functional atokous setae until quite late in metamorphosis, the atokous tissue is present in a reduced but still functional state until their loss, which occurs completely only during stage IV, after the eruption of the heteronereid setae.

Stage duration

In the calculation of the duration of each of the setal sac stages it has been assumed that the rate of oöcyte growth during each of the two growth phases is constant. However, in *Nereis diversicolor,* Clark and Ruston (1963) found that the rate of oöcyte diameter increase slowed down as the maximum diameter was approached. Although no evidence for such a rate change has been detected in *N. grubei,* the possibility that such a slow-down occurs in this species has not been completely eliminated. In that case, the calculated duration of stage IV, and possibly of stage III, would fall short of the true values. Good agreement of calculated growth rates with the timing of events in an animal which metamorphosed on the sea table lends credence to the calculations for *N. grubei.* It is possible that the different breeding habits of *N. diversicolor* might involve an additional change in oöcyte growth rate not found in epitokous species.

The appearance of heteronereid setae in the parapodia of young decapitated nereids has been reported in *Perinereis cultrifera* (Durchon, 1965) and in *Platynereis dumerilii* (Hauenschild, 1966). Durchon found that only 4 of 38 decapitated four-month-old specimens of *P. cultrifera* produced heteronereid setae. Hauenschild found that the parapodia of decapitated specimens of *P. dumerilii* with oöcytes under 45 μ underwent few externally visible metamorphic changes, with the exception of the occasional appearance of small, thin heteronereid setae. Since the heteronereid setae found in the parapodia of decapitated, gameteless *N. grubei* were recognized only after a careful re-examination of sectioned parapodia, their appearance may be more consistent than the initial reports indicated. The parapodial epidermis is thus competent to differentiate long before the stimulus to do so is normally supplied.

This study reveals that the first stage in heteronereid chaetogenesis occurs at the same time that the oöcyte growth rate increases in normal ontogeny (Schroeder, 1966). This fact supports the assumption that these two phenomena are controlled by the same hormone, which in *Nereis grubei* begins to wane more than two months before metamorphosis is completed and swarming takes place.

It is hoped that the description of these setal sac stages will engender greater precision in future work on nereid metamorphosis and its control, particularly when the response of isolated parapodia is used as an assay procedure.

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Summary

1. The development of the heteronereid setae during the metamorphosis of *Nereis grubei* is described. Four stages may be distinguished by the sequential appearance within the chaetogenic tissue of the several components of these compound setae. The characteristics of each stage are summarized in Table I.

2. Initiation of setal sac development results from the decapitation of even very young gameteless animals. At least a few cells of the parapodial epidermis are competent to produce morphologically complete setae in gameteless specimens, although both the cell and its product may be smaller than normal.

3. In females with oöcytes greater than about 50 μ in diameter, a detectable group of chaetogenic cells is often present within three days of decapitation; this tissue does not normally appear until the oöcytes are 95 μ in diameter.

4. Chaetogenesis already in progress may be accelerated by decapitation of animals during stage I and early stage II, when the oöcytes are under 140 μ in diameter.

5. Decapitation of animals with oöcytes greater than 140 μ in diameter did not accelerate chaetogenesis.

6. The duration of each setal sac stage has been calculated from the known oöcyte growth rate. Metamorphosis is estimated to occupy about 67 days.

7. The relationship between the setal sac stages and the stages already in the literature is examined.

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